Regression of established subcutaneous B16-F10 murine melanoma tumors after *gef* gene therapy associated with the mitochondrial apoptotic pathway

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Abstract: Novel treatment modalities, including gene therapy, are needed for patients with advanced melanoma. We evaluated whether the *gef* gene, a suicide gene from *Escherichia coli*, had a significant cytotoxic impact on melanoma *in vivo*. First, we used a non-viral gene delivery approach (pcDNA3.1/*gef*) to study the inhibition of melanoma cells (B16-F10) proliferation *in vitro*. Secondly, we used direct intra-tumoral injection of pcDNA3.1/*gef* complexed with jetPEI to deliver *gef* cDNA to rapidly growing murine melanomas. We demonstrated that *gef* gene not only has an antiproliferative effect on B16-F10 cells *in vitro*, but also induces an important decrease in melanoma tumor volume (77.7% in 8 days) *in vivo*. Interestingly, after *gef* gene treatment, melanoma showed apoptosis activation associated with the mitochondrial pathway, suggesting that the induction of this death mechanism may be an effective strategy for its treatment. Our *in vivo* results indicate that *gef* gene might become a suitable therapeutic strategy for patients with advanced melanoma.

Key words: apoptosis – caspase – *gef* gene – melanoma – mitochondria

Introduction

Melanoma represents only 4% of all skin cancers, but nearly 80% of skin cancer deaths, predominantly because of metastatic spread (1). Apart from surgery, treatment options for melanoma, particularly metastatic melanoma, are relatively limited. As melanoma is a highly therapy-refractory tumor, it demands effective therapies combinations (2). Suicide gene therapy has been proposed as a strategy for the treatment of intractable cancers and has been assayed in some clinical trials by itself or in combination with other therapies (tumor irradiation or chemotherapy). In melanoma, strategies to facilitate apoptosis by gene therapy may be an alternative or complementary strategy for its treatment (3) as it has been demonstrated that apoptosis deficiency is a critical factor for therapy resistance in this tumor (4).

Classical cancer suicide gene therapy employs genes encoding enzymes that convert non-toxic prodrugs into cytotoxic compounds (5). However, these prodrug systems have been assayed in melanoma both *in vitro* and *in vivo* with limited results (6,7). As an attractive alternative to this strategy, therapeutic genes that directly encode cytotoxic proteins could be used. In contrast to classical suicide genes that act by disrupting DNA synthesis (targeting only rapidly dividing cells) these new toxins may act killing both quiescent and rapidly dividing tumor cells and may be effective for aggressively growing tumors as well as for those that grow more slowly. Many genes encoding cytotoxic products have been evaluated as gene therapy approaches (8,9). The most recent experiences with genes expressing toxins from bacteria (10), from plants (11) or from bacteriophage (12) showed a high cytotoxic impact on tumoral cells derived from different tissues.

In this context, our group has developed last year a direct cancer gene therapy system based in the suicide gene named *gef*. The *gef* gene, member of a gene family with homologous cell-killing functions, encodes a membrane protein of 50 amino acids which is anchored in the cytoplasmic membrane by the N-terminal portion, whereas the C-terminal part is located in the periplasm (13). Although activation of this protein induces arrest of respiration and

death in bacterial cells the mechanism of action in tumoral cells is unclear. We have previously demonstrated that *gef* protein is able to induce changes in proliferation rate and differentiation degree of tumoral cells without having to use prodrugs (14,15). However, its possible *in vivo* application could not be demonstrated yet.

In this study, we have evaluated for the first time the potential use of the *gef* gene for the treatment of melanoma tumors *in vivo*. Moreover, based on the knowledge that the *gef* gene encodes a cytotoxic protein that binds to cell membranes, we analysed *gef*'s mechanism of action. We used the B16-F10 murine melanoma model because of its highly invasive and metastatic nature and the cationic lipids jetPEI to deliver *gef* cDNA to rapidly growing murine melanomas. Results obtained suggest that treatment with the *gef* gene significantly decreases tumor growth, inducing apoptosis in melanoma tumor cells by means of the mitochondrial pathway.

Methods

Cells and reagents

The B16-F10 murine melanoma cell line (CRL6475) was obtained from American Type Culture Collection and was grown in Dulbecco's modified eagle's medium (Sigma, St Louis, MO, USA), supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 40 mg/l gentamicin and 500 mg/l ampicillin (Antibióticos S.A, Madrid, Spain). Cells were maintained in monolayer culture at 37°C in an atmosphere containing 5% CO_2.

gef transfection in B16-F10 cells

The *gef* gene was amplified using specific primers (sense 5'-ATGAAGCAGCATAAGGCGATG-3' and antisense 5'-TTACTCGGATTCGTAAGCCGTC-3') under the following conditions: 94°C for 1 min, 35 cycles at 94°C for 1 min, 53°C for 30 s and 72°C for 30 s and 72°C for 10 min and was subcloned into the pcDNA3.1-TOPO vector (Invitrogen, Barcelona, Spain) following the manufacturers' instructions. The resulting plasmid pcDNA3.1/*gef* was transformed into the subcloning efficiency DH5 alpha chemically competent *Escherichia coli* (Invitrogen). The correct DNA sequence was confirmed by sequence analysis using the T7 primer. One day before transfection, confluent cells were seeded into six-well plates (2×10^5 cells/well). Briefly, a transfection mixture was prepared by adding 94 μl of the serum-free medium and 6 μl FuGENE-6 reagent (Roche Diagnostic, Barcelona, Spain). After 5 min of incubation at room temperature, 2 μg of plasmid DNA (pcDNA3.1/*gef*) were added (ratio 1:3). B16-F10 cells, yielding approximately 70% confluence, were transfected with *gef* gene-containing pcDNA3.1 vector. Cells were cultivated for 8 h at 37°C, and the medium containing transfection mixture was then replaced with the growth medium. A pcDNA3.1 plasmid in which the *gef* gene was absent was used as a negative control. The pcDNA3.1/green fluorescent protein (GFP) (provided by Dr. G. Ortiz) was used to optimize transfections conditions.

Proliferation assays

Parental and transfected cells growing in well plates were trypsinized after 24, 48, 72 and 96 h and collected. Cells were fixed and stained with 0.4% sulphorhodamine B/1% acetic acid. Cells previously washed with 0.1% acetic acid were left in 10 mM Trizma for 15 min at room temperature. Optical density was then determined using a Titertek multiscan (Flow, Irvine, CA, USA) colorimeter at 492 nm. Linearity of the SRB assay with cell number was tested for each B16-F10 cell stock before each cell growth experiment. B16-F10 cells transfected with empty vector were used in the proliferation assay as controls.

In vitro and *in vivo* expression of *gef* gene

Upregulation of mRNA expression of *gef* cDNA was determined by RT-PCR. RNA was extracted from transfected and parental cells with the RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA from tumor was obtained with the RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was generated by means of the Promega reverse transcription system using total cellular RNA (1 μg). PCR amplification of *gef* gene took place under the above-described conditions. RNA integrity was assessed by amplification of β-actin mRNA. Images were scanned and analysed using a Bio-Rad documentation system (Quantity One Analysis Software, Bio-Rad Laboratories, Barcelona, Spain). Relative *gef* mRNA expression was calculated as the ratio of *gef* to β-actin.

Annexin V and propidium iodide staining

Parental and transfected cells were washed twice with phosphate-buffered saline (PBS) and incubated in binding buffer containing annexin V-FITC (25 μg/ml) and propidium iodide (25 μg/ml) in the dark for 15 min at room temperature (Annexin V-FITC Apoptosis Detection Kit I; BD Pharmingen, San Diego, CA, USA). Then, binding buffer (500 μl) was added and cells were immediately processed with a FACScan flow cytometer. Microscopy analysis was carried out by Technical Services from the Granada University in a Leica DMI6000 (Heidelberg, Germany) confocal microscope with laser Argon/Krypton.

Assay for cytoplasmic mono- and oligonucleosomes

The Cell Death Detection ELISA Kit (Boehringer, Mannheim, Germany) was used for assessing apoptosis in transfected cells following the manufacturer's protocol. Parental and transfected cells (2×10^4) were lysed and the cell lysates were overlaid and incubated in microtitre plate

modules coated with antihistone antibody. Samples were then incubated with anti-DNA peroxidase followed by colour development with ABTS (2,2′-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)) substrate. Samples absorbance was determined with Titertek multiscan at 405 nm.

Measurement of mitochondrial membrane potential

Parental and transfected cells were washed twice with cold PBS and incubated with 40 nM DiOC6(3) for 15 min at 37°C. Then, cells were washed with ice-cold PBS and resuspended in 500 ml of PBS. Fluorescence intensities of DiOC6(3) were analysed on a FACScan (Becton Dickinson, San Jose, CA, USA) flow cytometer with excitation and emission settings of 484 and 500 nm, respectively.

Caspase activity assay

Caspase-9 and -8 activities were measured using caspase colorimetric assay kits (R&D Systems, Minneapolis, MN, USA). Briefly, parental and transfected cells were washed twice with cold PBS and resuspended in 50 μl of cold lysis buffer, incubated for 10 min and centrifuged for 1 min at 10 000 ***g*** to precipitate cellular debris. Assay was performed in triplicate on a 96-well plate following the manufacturers' protocol. Results are expressed as the fold increase in pcDNA3.1/*gef* treated cells over that of control cells. Etoposide (Sigma, St. Louis, MO, USA) (50 μmol/l) was used as positive control of caspase activities in B16-F10 cells.

Tumor induction and measurement

For *in vivo* study, female C57BL/6 mice (Scientific Instrumentation Centre, Granada University, Granada, Spain) were used. All mice (weighing 25–30 g) were maintained in a laminar air-flow cabinet at a room kept at 37°C temperature and 40–70% humidity with a 12-h light/dark cycle under specific pathogen-free conditions. All studies on animal models were approved by the Ethical Committee of the Medical School of Granada University and performed according to its guidelines. Tumors were induced by subcutaneous injection of 5×10^5 B16-F10 cells into the left flanks of C57BL/6 mice. Tumors were allowed to grow to the appropriate size (75 mm^3) before treatment (ideal minimal size for intratumoral injection). After reaching this volume (treatment day 0), tumors were measured at periodic intervals following treatment using a digital caliper by measuring the longest diameter (a) and the next longest diameter (b) perpendicular to (a). Using these measurements the tumor volume was calculated by the formula $V = ab^2 \times \pi/6$.

Intra-tumoral plasmid treatment

In vivo JetPEI (Polyplus-transfection Inc., New York, NY, USA) was used as a transfection enhancer reagent. PEI/DNA complexes with a ratio of 1:6 were prepared in a solution of 10% w/v glucose. This was carried out in a two-step procedure for the preparation of a standard quantity of 20 μg of PEI/DNA complex, according to the manufacturer's instructions. Tumors were then treated intra-tumorally with pcDNA3.1/*gef* plasmid. The pcDNA3.1 LacZ plasmid was used to normalize transfection efficiency. Moreover, control groups (without treatment and treated with empty vector) were included. Treatments were administered during 14 days. Comparative study between treated and non-treated groups was realized during the first 8 days because of the high rate of mortality in control group.

Histological analysis

Tumors were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and cut into 3–5 μm sections. Cells were immunofluorescently labelled with primary antitubulin mouse monoclonal antibody (1:500) (Sigma) followed by Texas Red dye-conjugated affinitPure Goat Anti-Mouse IgG + IgM (1:500) (Jackson ImmnoResearch Laboratories, West Grove, PA, USA). The presence of apoptotic cells within the tumor sections was evaluated by the TUNEL technique using the In Situ Cell Death Detection Kit Fluorescein (Roche, Mannheim, Germany) according to manufacturers' recommendations. Cell nuclei were counterstained with DAPI. Per cent apoptosis (apoptotic index) was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field (5 high power fields/slide). Immunohistochemical analyses of caspases were realized using antiactive forms of caspase-9 (1:50) (Cell Signaling Technology, Inc, Danvers, MA, USA) and caspase-8 (1:100) (Imgenex, San Diego, CA, USA). FITC-conjugated antirabbit secondary antibody at room temperature for 1 h was used for the detection. Cell nuclei of cultures were counterstained with DAPI. Fluorescence images were captured using an Olympus DP11 microscope with a Nikon Eclipse Ti (Nikon Instruments Inc., New York, NY, USA) digital imaging system.

Transmission electron microscopy

Melanoma tumors grown in mice were collected, cut up into small pieces and immediately fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 1 h. After postfixation with 1% OsO_4 in cacodylate buffer (room temperature, 2 h), sections were dehydrated through graded ethanol concentrations with a final propylene oxide dehydration. Samples were then embedded in Epon 812 resin. Ultrathin sections, were stained with uranyl acetate and lead citrate and examined in a Hitachi H7000 transmission electron microscope (TEM, Hitachi High-Technologies Corporation, Tokyo, Japan).

Statistical analysis

SPSS 14 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Results were compared by using

the Student's *t*-test. All data are expressed as mean ± SD. Differences were considered statistically significant at a *P*-value of <0.05.

Results

Expression of *gef* gene and inhibition of cell growth in B16-F10 cells in culture

In vitro evaluation of *gef* gene expression was performed by RT-PCR. As shown in Fig. 1a, an amplification fragment of 153 bp was found in B16-F10 cells transfected with pcDNA3.1/*gef* for different time periods, indicating the effectiveness and ability of the construction in order to be used in the subsequent experiment. To demonstrate the integrity of the RNA preparations, PCR was performed using β-actin primers. Analysis of the bands, normalized by comparison with the β-actin signal, showed a progressive increase of *gef* expression; this was 3.9- and 4.5-fold higher at 48 and 72 h vs B16-F10 cells at 24 h and was maximal at 96 h after transfection (sixfold higher vs B16-F10 cells at 24 h). Previously, cell transfection was optimized by pcDNA3.1/GFP (Fig. 1b). As shown in Fig. 1c, the B16-F10 cells transfected with pcDNA3.1/*gef* showed a significant and time-dependent decrease in growth. Twenty-four hours after transfection a 28% decrease in growth rate versus control cultures was observed. The decrease in proliferation was 45.5% at 48 h. The main decrease in proliferation rate occurred at 72 h and 96 h, when similar ratios of growth decrease were observed (64.6% and 69.7%, respectively). In contrast, the growth of B16-F10 cells transfected with the empty vector (control group) was similar to that of the parental cells.

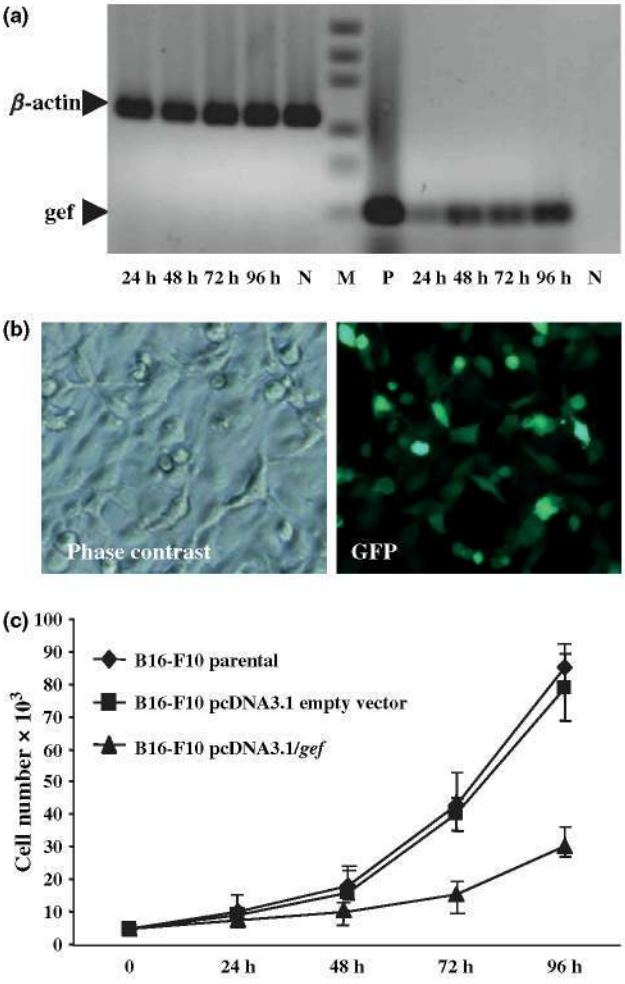

Figure 1. *gef* gene expression and growth rate inhibition in B16-F10 cells. (a) RT-PCR showing *gef* gene expression in B16-F10 transfected cells at different time periods. The integrity of the RNA was demonstrated using β-actin primers. N, negative control (B16-F10 non-transfected cells); M, molecular weight; P, positive control (pcDNA3.1/*gef*). (b) Representative photomicrograph (phase contrast and fluorescent images) of B16-F10 cells transfected with pcDNA3.1/GFP to optimize transfections conditions. (c) Growth of B16-F10 cells expressing *gef* was detected by sulphorhodamine B assay. Cells transfected with pcDNA3.1/*gef* showed a clearly decreased growth rate compared to the control cells and cells transfected with pcDNA3.1 empty vector ($P < 0.05$). Data represent the mean ± SD of four independent experiments.

Expression of *gef* gene-induced apoptosis in B16-F10 cells

Apoptotic rates of B16-F10 cells untreated or transfected with empty vector revealed no significant difference. Only 8.7% of the pcDNA3.1/*gef* transfected cells showed apoptosis after 24 h. However, at 48 and 72 h a significant increase was found (19% and 34%, respectively) (Fig. 2a). At 96 h apoptosis was similar to that found at 72 h (data not shown). These results indicated the ability of *gef* gene to stimulate apoptosis in B16-F10 melanoma cells after *in vitro* transfection. The induction of apoptosis by *gef* gene was also evident by confocal laser-scanning microscopy (Fig. 2b). Furthermore, to confirm whether the growth inhibitory effects of *gef* gene are related to the induction of apoptosis, we used an ELISA-based assay. Amounts of cytoplasmic oligonucleosomes (an indicator of apoptosis) increased between 24 and 96 h after *gef* transfection as compared with untreated cells. As shown in Fig. 2c, the strongest enrichment factor was obtained at 72 and 96 h (3.6 and 5.4, respectively). These results provide convincing data that up-regulation of *gef* induces apoptosis in B16-F10 cancer cells.

Gef gene therapy-induced modulation of mitochondrial membrane potential and caspase-9 activation

To determine if induced apoptosis by *gef* gene in B16-F10 cells is mediated via the mitochondrial pathway, mitochondrial membrane integrity was measured by DiOC6(3) dye staining. As shown in Fig. 3a, a significant decrease in

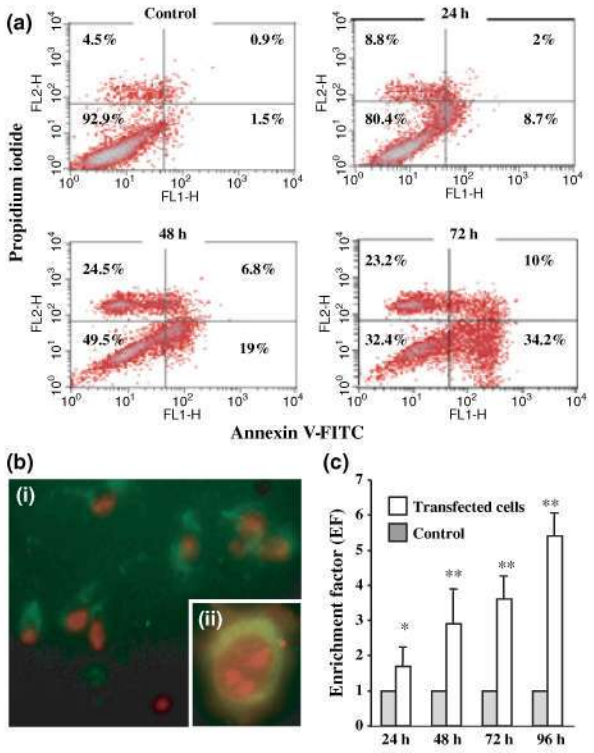

Figure 2. Analysis of apoptosis induction by *gef* gene in B16-F10 cells. (a) Fluorescence-activated cell sorting. Cells were stained with annexin V and propidium iodide (PI) to evaluate apoptotic cell death, as described in Methods. These data are representative results from four separate experiments. (b) Annexin V-FITC staining and confocal microscopy. The annexin V-FITC fluorescence localized at the periphery of the cells consequent to the translocation of phosphatidylserine (PS) residues from the inner leaflet of the plasma membrane to the outer leaflet. B16-F10 cells 48 h after transfection (a). Cell nuclei were counterstained with PI before examination under a confocal laser-scanning microscope. A stronger binding was observed when the study was carried out 96 h after *gef* transfection (b). The experiment was performed three times with identical results. Magnification: (a) 20×; (b) 40×. (c) ELISA apoptosis assay of cytoplasmic nucleosomes. The specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated using the formula: mean of absorbance of transfected cells/mean of absorbance of control cells = enrichment factor (EF). The EF was calculated relative to the control value (=1) of untreated cells. $*P < 0.05$; $**$ $P < 0.01$ compared to control cells.

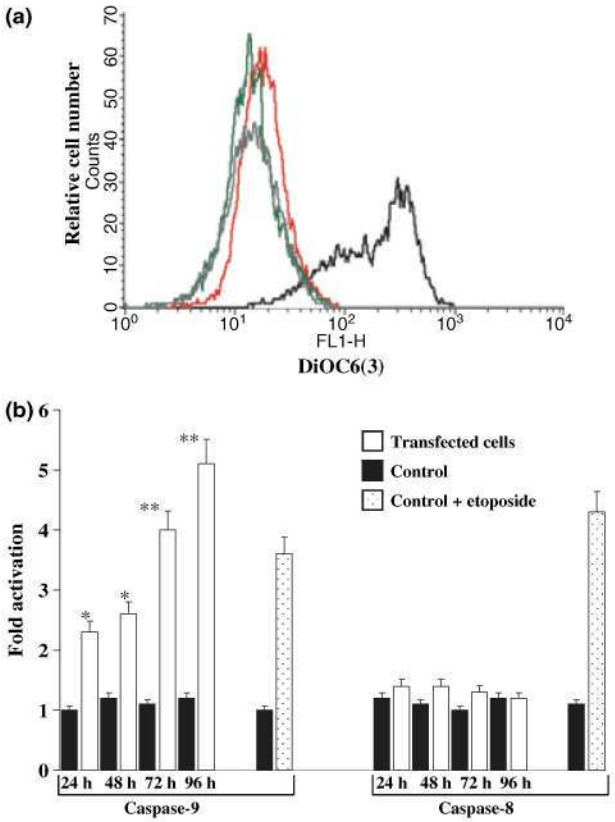

Figure 3. Mitochondrial membrane potential and caspase activity. (a) Reduction of mitochondrial transmembrane potential ($\Delta\psi_m$) in B16-F10 cells (black) after 24 h (red), 48 h (grey) and 72 h (green) of *gef* transfection. DiOC6(3) was added to the culture medium during the last 15 min of treatment at a final concentration of 40 nM. The fluorescence intensity of DiOC6(3) was analysed by flow cytometry. Data shown are representative of three independent experiments. (b) Caspase-9 and -8 activities were determined (as described in Methods) in pcDNA 3.1/*gef* transfected B16-F10 cells at indicated time points in comparison with parental cells (percentage values). Etoposide treatment (6 h) was used to demonstrate caspase 8 and caspase 9 activities in B16-F10 cells. Experiments were performed four times with identical results. $*P < 0.05$; $**$ $P < 0.01$ compared to control cells.

membrane potential was detected in transfected cells at 24, 48 and 72 h compared with parental cells (control) indicating a mitochondrial membrane permeability increase after *gef* gene treatment. At 96 h membrane potential modulation was similar to that found at 72 h (data not shown). Caspase-9 activity was induced after *gef* gene treatment in B16-F10 cells. Although its activity was modulated at different times, the largest increase was observed at 72 and 96 h (three- and 4.1-fold; respectively). The caspase-8 activity remained unchanged (Fig. 3b). These data support the hypothesis that *gef* gene-induced apoptosis through the mitochondrial-mediated pathway.

Gef gene effects on melanoma growth *in vivo*

The potential of *gef* gene to promote tumor cell killing *in vivo* was evaluated by direct injection of the plasmid complexed with jetPEI in B16-F10 subcutaneous mice tumors. Figure 4 shows that *gef* gene was able to inhibit tumor growth. During the first 2 days following treatment, tumor volume decreased by 40.4% in the *gef* gene treated group, as compared with the control group. On post-treatment days 4 and 6, the observed reduction was 45.2% and 54.3% respectively. Following a similar trend, a 77.7% volume reduction was observed on day 8. After this time, the control group showed a high mortality rate (Fig. 4). Mice treated with *gef* gene showed no evidence of systemic toxicity (i.e. animal death, loss of body weight, other tissue damage

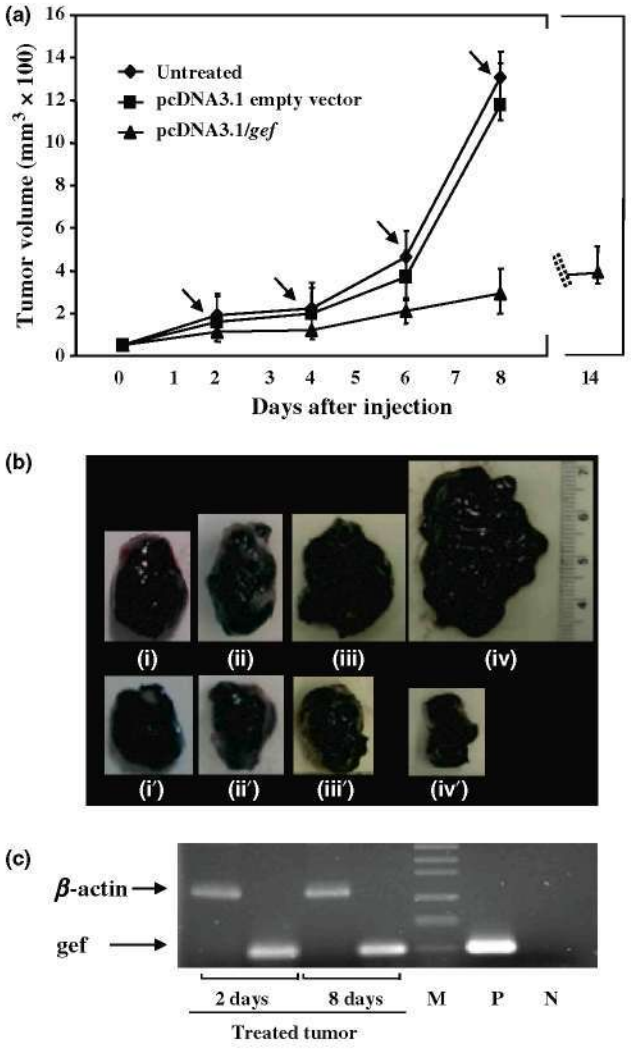

Figure 4. Effect of direct intra-tumoral injection of the *gef* gene on the growth of subcutaneous tumor induced by B16-F10 tumors cells in mouse. (a) Tumor volume variation after *gef* gene treatment. Time of injection is indicated through arrows; treatment began (day 0) when tumors had reached a volume of 75 mm^3. The plot shows a significant tumor volume reduction in the treated group (n = 14) as compared with the control groups, transfected with empty vector (n = 7) and without treatment (n = 7). (b) Representative gross appearance of tumors excised from mice sacrificed during treatment at 2 (a'), 4 (b') 6 (c') and 8 (d') days and tumors obtained from mice without treatment at the same time intervals (a, b, c and d, respectively). (c) Determination of *gef* gene expression in tumor mice after 2 and 8 days of pcDNA3.1/*gef* treatment. The integrity of the RNA tissue used was determined using *β-actin* primers. M, molecular weight; P, positive control (pcDNA3.1/*gef*); N, negative control (mouse tumors without treatment).

or changes in behaviour or aspect). After empty vector injection with jetPEI, no tumor growth modifications were observed (Fig. 4a,b). RT-PCR was performed to ensure *gef* gene expression in all treated tumors. Figure 4c shows a comparison of *gef* gene expression in melanoma tissue between days 2 and 8 after the treatment.

gef gene-induced apoptosis in melanoma cells *in vivo*

To determine the *gef* expression efficiency to induce apoptosis *in vivo* we analysed established subcutaneous B16-F10 tumors with a TUNEL reaction mixture. As showed in Fig. 5a, the number of apoptotic cells (green) was significantly higher in tumors treated with pcDNA3.1/*gef* in comparison with the tissue control. Analysis of the melanoma sections showed a progressive increase of per cent apoptosis which was maximal at 8 days after treatment (Fig. 5b). To examine possible caspase-9 or -8-activation by *gef* gene treatment we used immunohistochemistry. Weak or absent expression of caspase-8 protein was detected in tissue samples (data not shown). However, all samples of melanoma tissue treated with pcDNA3.1/*gef* at different times showed a clear caspase-9 activation, with the strongest staining observed from the 4th day of treatment until the end of the experiment. Caspase-9 expression was not seen in any of the controls or in melanoma tissue treated with the empty vector (Fig. 5c).

Transmission electron microscopy

To further investigate the nature of *gef* gene-mediated cytotoxicity, B16-F10 mouse melanoma-induced tumors treated with *gef* gene *in vivo* were analysed by TEM. The control tissue showed giant malignant cells with an intact cell membrane and single- or multiple-nuclei cells. The first identifiable morphological change after treatment was the pronounced swelling in the mitochondria seen within 2 days of treatment. Mitochondria in control cells and cells treated with vehicle alone remained unaffected. Forty-eight hours later, apoptosis ultrastructural characteristics, such as chromatin condensation, crescent formation and margination were seen by electron microscopy in the treated melanoma, but not in the control group. Similar pictures were observed 6 and 8 days after treatment (Fig. 6).

Discussion

New treatment strategies for malignant melanoma are urgently needed because conventional approaches like chemotherapy and radiation have little impact on patient survival in the advanced stages of the disease. As a promising alternative, gene therapeutic strategies based on suicide gene expression in tumor cells have been developed. In this study, we have demonstrated the *in vivo* potential use of the *gef* cDNA as a suicide gene in a new melanoma gene therapy approach.

To date, classical suicide gene therapy systems have not guaranteed the successful treatment of melanoma and have

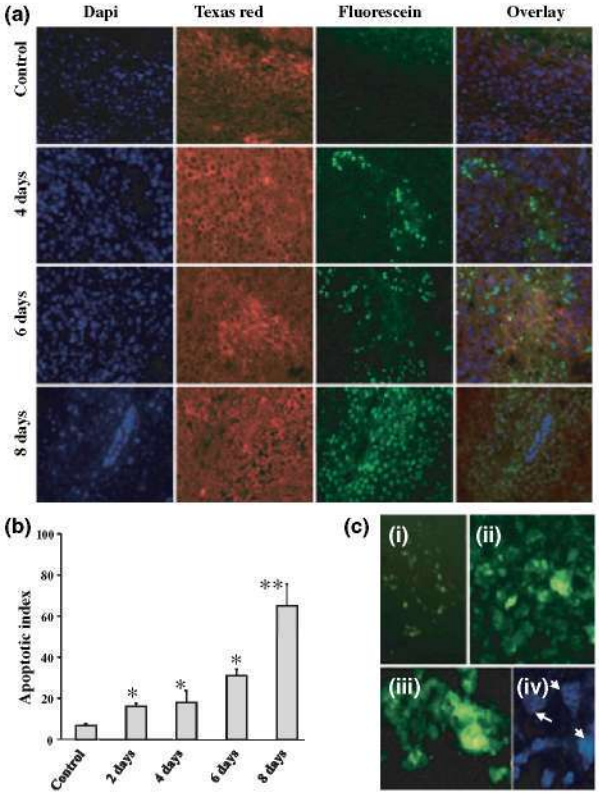

Figure 5. Histological evaluation of apoptosis and caspase activity of melanoma cells *in vivo*. (a) Representative photographs of tumor sections showing TUNEL-positive cells (green). Sections were counterstained with DAPI (blue) and anti-tubulin (red). Apoptotic cells increased in tumors treated with pcDNA 3.1/*gef* at 2 (data not shown), 4, 6 and 8 days compared to untreated tumors (control) (×20). (b) Per cent apoptosis in each group. Values were expressed as mean ± SD. Level of significance compared to control cells: $*P < 0.05$; $**P < 0.01$. (c) Caspase expression in melanoma tumor was detected using immunofluorescence staining. Microscopic analysis showed that melanoma tissue after treatment with pcDNA3.1/*gef* was strongly caspase-9-positive. Untreated tumors (a) and melanoma tumor after 4 (b) (40×) and 6 (c) (60×) days of treatment. Cell nuclei counterstained with DAPI showed that some cells displayed apoptotic morphology and nuclear segmentation (arrows) (d, 60×). All data were obtained from the study of at least three tumors.

induced only a partially positive response (16,17). One of the main limitations of these indirect action systems is the need to use prodrugs (18).The use of genes encoding toxins avoids the administration of a prodrug, eliminating its side-effects, its bioavailability limitations and the consecutive applications of vector and prodrug. Moreover, these genes can be directly expressed in the cytosol of the target cells, thus overcoming the problems (cytotoxicity, internalization efficiency and resistance acquired by cancer cells) originated by their use as components of immunotoxins or recombinant chimeras (19). In melanomas, viral genes encoding toxins such as viral protein R and some plant

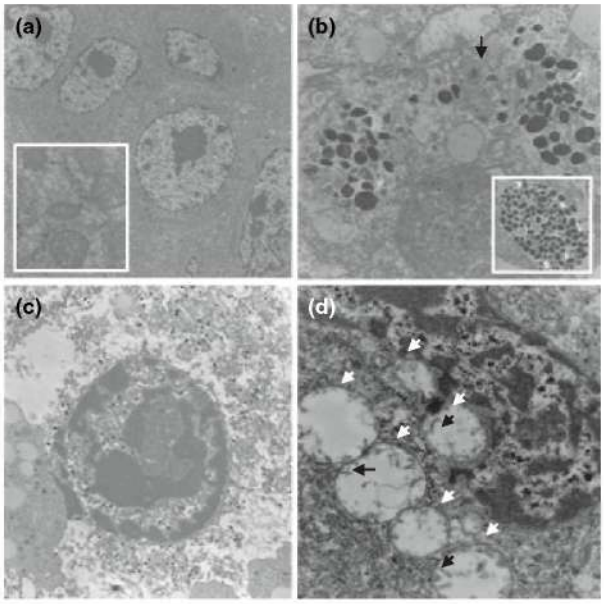

Figure 6. Transmission electron microscopy of melanoma tumors without treatment showed typical tumor cells with polygonal shape, large nucleus, light cytoplasmic complexion containing well preserved organelles (a) including mitochondrias (A insert) (1100×) and a large amount melanosomes (B insert) including premelanosomes (arrows) (b) (×4000). Representative photomicrograph of melanoma treated with pcDNA3.1/gef (6 days) showing ultrastructural characteristics of apoptosis such as chromatin condensation, crescent formation and margination (c) (×6300). Note, the nucleus near of the swollen mitochondrias (white arrows) with disrupted cristae (black arrows) (d) (12000×). Data were obtained from the study of at least three tumors.

genes such as saporin (SAP) have been applied with a significant result to induce tumoral cell death (20,21). Our previous results *in vitro* showed that *gef* is effective in melanoma MS-36TG cells, modulating their proliferation capacity, differentiation degree and tumor malignancy (22). In the present study, we have demonstrated that the transfection of the pcDNA 3.1/*gef* not only inhibits *in vitro* melanoma proliferation but also it is highly toxic for tumors *in vivo*. The *gef* gene treatment induced a significant decrease in tumor growth (77.7% relative volume reduction after 8 days of treatment), an effect that was clearly improved upon repeated administrations. Experimental treatment with HSV-tk/GCV (23) or more recently with the SAP gene (21) in the same tumor type, induced a 40–50% and 67% relative volume reduction, respectively. McCray et al. (20), who used the Vpr gene integrated in the pcDNA3.1 (100 μg) vector in melanoma tumor from B16-F10 culture cells, described an 86% of tumor volume reduction which required 25 days of treatment. Therefore, the main advantage of the *gef* gene is not only its efficacy in melanoma cells but also the shorter latency for effective antitumoral action.

Although we have demonstrated the cytotoxic effect of *gef* gene in cancer cells, the specific mechanism of action has remained unclear so far. In prokaryotic cells, the *gef* gene diminishes the membrane potential, leads to membrane leakiness and also induces morphological changes (24). Eukaryotic cells fundamentally differ from prokaryotic cells in terms of their cellular structure, organization, metabolism and membrane composition. Nevertheless, as the eukaryotic endomembrane system arose in an ancestral prokaryotic lineage (25) *gef* gene might act in cell organelle membranes. Recently, it was demonstrated that breast cancer cells growth was inhibited by bacteriophage λ-holin, a protein that can permeabilize the bacterial membrane (12). Our results showed that 48 h after induction B16-F10 cells become multinucleated, in some cases extensively vacuolated and finally detached from the culture dish surface. Experiments with annexin, confocal laser-scanning microscopy and nucleosomes clearly showed that the *gef* gene is able to induce apoptosis in a time-dependent manner. These results are similar to those obtained with the SAP gene which also induces programmed cell death and direct DNA fragmentation in B16-F10 cells (21). Interestingly, the pronounced clinical chemoresistance of melanoma is strongly suggestive of an inactivation of apoptotic programmes. Defects in proapoptotic signalling pathways and enhancement of antiapoptotic pathways may synergistically contribute to this apoptosis deficiency (26). Immunohistochemical analysis by TUNEL assay revealed that pcDNA3.1/*gef* treatment significantly increased apoptosis in established subcutaneous B16-F10 tumors *in vivo*. The incidence of apoptosis in the tumor almost corresponded to the effect of tumor growth inhibition, suggesting that our experimental treatment resulted in tumor regression by significant augmentation of apoptosis.

Apoptosis may occur via death-receptor dependent (extrinsic) or mitochondrial (intrinsic) pathways. The extrinsic pathway is triggered by the activation of death receptors, such as Fas and TRAIL receptors (DR4, DR5) activating initiator caspase-8, which then cleaves executioner caspase-3. The mitochondrial pathway of cell death is mediated by Bcl-2 family proteins, which disrupt the mitochondria membrane potential and result in release of apoptogenic factors, such as cytochrome *c*, from the mitochondria into cytosol; in turn, these factors would form an apoptosome with apoptosis activating factor 1 and caspase-9 (27). Treatments modulating apoptosis phenomenon, for example with bcl-2-targeted antisense, are a promising new strategy in melanoma (28). Assays with drugs such as hydroquinone or thiobenzanilides in this tumor type have demonstrated an action mechanism related to caspase-9 activation (29,30). This tumoral cellular injury mediated by caspases may also be induced by suicide genes and it may be relevant in relation to their application in tumors. In fact, CD/5FC system induces apoptosis in human malignant glioma cells by the activation of caspases-3 and -9 but not caspase-8 (31) while a certain modification, the bifunctional *E. coli* cytosine deaminase and uracil phosphoribosyltransferasefusion, is able to induce caspase-3 activation only (32). HSV*tk*/GCV activates caspase-3, -8 and -9 in rat bladder carcinomas (33) and a variant, the thymidylate kinase, induces apoptosis in Jurkat cells by activation of caspase-3 only (34). Our studies in B16-F10 cells expressing *gef* showed alteration of the mitochondrial membrane integrity suggesting that apoptosis is mediated by the mitochondrial pathway. This hypothesis is supported by the caspase-9 activity increase in B16-F10 transfected cells. Moreover, the mitochondrial transmembrane potential is altered in most of the cellular population, supporting the hypothesis on the possible effect of *gef* once it is released from the apoptotic cells. The mitochondrial-mediated apoptotic pathway is strongly supported by our ultrastructural findings in the induced B16-F10 tumors in mice which showed dilated mitochondria with disrupted cristae. Moreover, the *in vivo* assay shows that caspase-9 activity increases significantly after *gef* gene treatment, supporting the participation of a mitochondrial-mediated apoptotic pathway in our gene therapy system. However, we can not exclude the possible participation of other apoptosis-mediated molecule in treated B16-F10-induced tumors such as endonuclease G, Smac/DIABLO and HtrA2 (35). Further studies are required to elucidate the exact mechanisms involved.

We have reported the successful use of the *gef* gene as an anticancer gene therapy system, not only in melanoma cells in culture but in melanoma tumors *in vivo*. Our *in vivo* experiments show that *gef* gene has a rapid and efficient activity in relation to tumor volume decrease. However, *gef* gene binds to the mitochondrial membrane and its activity is not tumor-specific. Therefore, it will be necessary to create this specificity as in most of the toxic genes used in gene therapy (8–12). We have injected intra-tumorally the pcDNA3.1/gef plasmid to observe its activity in melanoma cells. Adenoviral vectors modified by attaching tumor-specific promoters should be used to assay metastatic melanoma treatment. Currently, we are using specific enhancer/promoter genes (such as tyrosinase) (36), new vectors (such as ReCon) (37) and combined therapy with cytotoxic drugs (38) to improve the tumoral response against *gef* gene. Moreover, it will be necessary to demonstrate the apoptosis induction in human melanoma by the extopic *gef* gene expression. In summary, our results suggest that *gef* is a suicide gene candidate for oncologic *in vivo* applications and that it may contribute to eradicate tumor mass in combination with surgery or classic radio- or chemotherapy.

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